

## Enterotoxin-encoding genes in *Staphylococcus aureus* from buffalo milk<sup>1</sup>

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**ABSTRACT.**- Moura E.O., Rangel A.H.N., Macêdo C.S., Urbano S.A., Novaes L.P. & Lima Júnior D.M. 2019. **Enterotoxin-encoding genes in *Staphylococcus aureus* from buffalo milk.** *Pesquisa Veterinária Brasileira* 39(8):587-591. Universidade Federal de Alagoas, Campus Arapiraca, Av. Manoel Severino Barbosa s/n, Bom Sucesso, Arapiraca, AL 57309-005, Brazil. E-mail: [juniorzootec@yahoo.com.br](mailto:juniorzootec@yahoo.com.br)

This paper investigated the occurrence of *Staphylococcus aureus* and the detection of enterotoxin-encoding genes of these strains in milk collected from 30 Murrah buffaloes used to produce dairy products in Brazil. A total of 68 strains of *Staphylococcus aureus* were found as identified by conventional laboratory tests, and thus screened for *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh* and *sei* enterotoxin-encoding genes by polymerase chain reaction (PCR). Twelve strains containing enterotoxin-amplified genes were found, with higher expression for the *sei* and *seh* genes. These results can be attributed to animal health and inadequate cleaning of the equipment, indicating the need for better quality control in animal production and health lines. The results of this study with the presence of pathogens and their enterotoxigenic potential indicate a source of food poisoning, as well as being a pioneering study in the detection of new enterotoxins for buffalo milk.

**INDEX TERMS:** Enterotoxin, encoding genes, *Staphylococcus aureus*, buffalo, animal origin food, food microbiology, mastitis, milk quality, bovine.

**RESUMO.**- [Genes codificadores de enterotoxinas em *Staphylococcus aureus* no leite de búfalas.] Este estudo investigou a ocorrência de isolados de *Staphylococcus aureus* e a detecção de genes que codificam a enterotoxigenicidade dessas cepas em leite de búfala utilizado na produção de laticínios no Brasil. As amostras foram coletadas em 30 búfalos da raça Murrah, identificado por testes laboratoriais convencionais, foram identificados um total de 68 cepas de *S. aureus* e rastreados para os genes que codificam a enterotoxina *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh* and *sei* por reação em cadeia da polimerase (PCR). Doze cepas contendo genes da enterotoxina foram amplificadas, com maior expressão para os genes *sei* e *seh*. Esses resultados podem ser atribuídos à saúde animal e à higiene inadequada do equipamento, indicando

a necessidade de melhor controle de qualidade nas linhas de produção e saúde animal. Os resultados desta pesquisa, com a presença de patógenos e seu potencial enterotoxigênico, indicam uma fonte de intoxicação alimentar, além de ser uma pesquisa pioneira na detecção de novas enterotoxinas para o leite de búfala.

**TERMOS DE INDEXAÇÃO:** Genes codificadores, enterotoxinas, *Staphylococcus aureus*, búfalos, produtos de origem animal, microbiologia de alimentos, mastite, qualidade de leite, leite de búfala, bovinos.

### INTRODUCTION

*Staphylococcus aureus* is among the most prevalent etiological agents of subclinical mastitis in different animal species, and it stands out for having high pathogenic potential and virulence. The *S. aureus* microorganism is equipped with pathogenic mechanisms that damage the host's tissue and facilitate colonization; moreover, *S. aureus* is one of the major causative agents of foodborne diseases worldwide and contributes to high food poisoning incidence (Silva et al. 2013). As it is frequently isolated in raw milk, it is considered responsible for an increase in milk somatic cell count

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(SCC), and consequent reduction in productivity while also compromising the milk's nutritional composition (Silva et al. 2000). The enterotoxins produced by *S. aureus* (known as staphylococcal enterotoxins - SEs) are heat-resistant, which preserves their biological activity after milk pasteurization or ultra-high-temperature processing. This fact explains disease outbreaks where milk and milk products have been directly implicated via SEs (Rosa et al. 2015).

Therefore, the objective of this study was to investigate the occurrence of *Staphylococcus aureus* in raw buffalo milk and also assess the presence of staphylococcal enterotoxin-encoding genes.

## MATERIALS AND METHODS

**Ethics statement.** The project for this study was submitted to the Ethics Committee in Animal Use (CEUA), receiving opinion number 007/2015, being free and approved for implementation from the legal point of view, according to Law No. 11,794, 2008.

**Sample collection and Staphylococcus identification.** Raw milk samples for *Staphylococcus* spp. isolation which had been collected from 30 Murrah buffaloes in a commercial herd operation located in Taipu (Rio Grande do Norte - RN, Brazil) were put into a refrigerator at 4°C for 8 hours. Group 1 consisted of 15 randomly selected animals in early lactation stage with production  $\geq 15$ kg milk/day, and Group 2 consisted of 15 animals in late lactation stage with production  $\leq 8$ kg milk/day. Buffalo cows were kept in pasture with access to concentrate supplementation according to their productivity, and were milked twice daily at 5:00 a.m. and 3:00 p.m. by a mechanized system (milking parlor). Milk was collected from all mammary quarters directly from the animals' teats. The samples were collected in sterile bottles (20mL) after application of hygienic procedures consisting of the first jets of milk, asepsis of the ceilings by a chlorinated solution, drying of the ceilings with a disposable towel, followed by disinfection of the posterior end of the ceiling and sphincter with cotton soaked in 70% alcohol (v/v), with the handler using disposable gloves at all times. The samples were then transported under refrigeration to the Quality Control Laboratory and the Microbiology Laboratory of Food Engineering at the "Universidade Federal do Rio Grande do Norte", Natal/RN, Brazil.

For *Staphylococcus aureus* isolation, 25mL of milk was taken from each sample and diluted in 225mL of sterile peptone water (0.1% wt./vol.). Decimal serial dilutions ( $10^{-1}$  and  $10^{-2}$ ) of each sample were then made from this stock solution, and 1-mL aliquots were plated in duplicate into Baird-Parker agar enriched with egg yolk emulsion and 50% potassium tellurite at 1.0% and were then incubated at 36°C for 48h for colony growth (Silva et al. 2001). Next, 5 typical and 5 atypical colonies were randomly selected and inoculated into brain heart infusion broth at 36°C for 24h. Isolated colonies were used for additional identification tests such as Gram staining (Koneman et al. 2001), as well as assays for catalase and coagulase production (Silva et al. 2001). Tests for acetoin production (Voges Proskauer test), 0.04IU bacitracin resistance, and the fermentation of mannitol, maltose, and trehalose sugars were performed according to Koneman et al. (2001). In addition, an acriflavine resistance test was performed according to Brito et al. (2002).

**Antimicrobial susceptibility testing.** An antibiotic sensitivity test was also performed by the VidaVet laboratory through the disk diffusion method in agar for the identified samples, according to the methodology recommended by the National Committee for Clinical Laboratory Standards (NCCLS 2003), using the bases of commercial products available for clinical treatments of samples from cows with

mastitis and different antimicrobials of samples from each buffalo. For this, 17 active ingredients were used in these sensitivity tests, as these drugs are the most used by breeders and all commercial antibiotics that the veterinarian can choose as the one which best applies to the inflammatory situation of each animal. The antibiotics applied for the antibiogram were: PMN = Novobiocin (40µg) + Penicillin G procaine (40µg), CEQ = Cefquinome (30µg), AMO = Amoxicillin (10µg), DUL = Danofloxacin (5µg), CTF = Ceftiofur (30µg), CL = Cephalexin (30µg), CN = Gentamicin (10µg), TE = Tetracycline (30µg), AM = Ampicillin (10µg), ENO = Enrofloxacin (5µg), NEO = Neomycin (30µg), SUT Sulfatrim (25µg), AMC = Ampicillin + Colistin (30µg), CNM = Cefalonium (30µg), ACA = Amoxicillin (20µg) + clavulanic acid (10µg), OXA = Oxacillin (1µg), and CIP = Ciprofloxacin (5µg).

**Detection of enterotoxin-encoding genes.** Genomic DNA of isolates was extracted by thermal lysis according to the protocol recommended by Pacheco et al. (1997). After extraction, the DNA was kept at -20°C and quantified in a spectrophotometer (Nanodrop 2000 Termo Cientific®, Wilmington/DE). Polymerase chain reactions for the detection of *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, and *sei* genes (Johnson et al. 1991, Mehrotra et al. 2000, Jarraud et al. 2002) consisted of a mixture of 1µL of primer (Invitrogen, Carlsbad, California) (Table 1), 12.5µL of 1X Master Mix (dNTP, MgCl<sub>2</sub>, and Taq DNA polymerase; Promega, Madison/WI), 5.5µL of nuclease-free water (Promega), and 5µL of total DNA for a final volume of 25µL. Amplification for all genes was performed in a thermocycler (BIO-RAD, Hercules/CA) with the following cycles: initial denaturation for 5 minutes at 94°C and then 30 cycles at 94°C for 2 minutes (denaturation) and 72°C for 1 minute (extension). The various temperatures used in the annealing step are shown in Figure 1. Final extension was performed at 72°C for 5 minutes (Rall et al. 2010).

The PCR-amplified samples were analyzed by electrophoresis for 50 minutes at 110V through a 1.5% agarose gel (Invitrogen, USA) in 0.5X TBE (0.09 M Tris-HCl, 0.09M boric acid, 2mM EDTA,

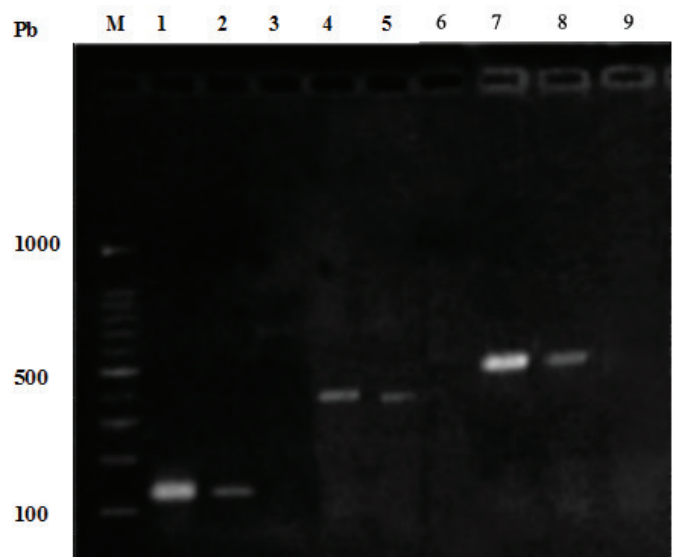


Fig.1. Agarose gel containing the amplified PCR products from enterotoxin-encoding genes of *Staphylococcus aureus*. M = 1,000bp DNA ladder, 1 = positive control for *sea* (120 bp), 2 = positive sample for *sea* (120 bp), 3 = negative control for *sea*, 4 = positive control for *seh* (376 bp), 5 = positive samples for *seh* (376 bp), 6 = negative control for *seh*, 7 = positive control for *sei* (577bp), 8 = positive samples for *sei*, and 9 = negative control for *sei*.

**Table 1. Primers and temperature used for the detection of staphylococcal enterotoxin genes**

Gene	Primer	Sequence	Base pair	Annealing temperature	Reference
<i>sea</i>	SEA-F	ttggaacggttaaaacgaa	120	52.3°C	Johnson et al. (1991)
	SEA-R	gaaccttccatcaaaaaca			
<i>seb</i>	SEB-F	tcgcatcaaaactgacaaacg	478	52.3°C	Johnson et al. (1991)
	SEB-R	gcaggtactctataagtcc			
<i>sec</i>	SEC-F	gacataaaagctaggaattt	257	52.3°C	Johnson et al. (1991)
	SEC-R	aaatcggattaacattatcc			
<i>sed</i>	SED-F	ctagtttgtaatatctct	317	51.1°C	Johnson et al. (1991)
	SED-R	taatgctatatcttaggg			
<i>see</i>	SEE-F	agggtttttcacaggtcatcc	209	50°C	Mehrotra et al. (2000)
	SEE-R	ctttttttctcggtcaatc			
<i>seg</i>	SEG-F	aattatgtgaatgctcaaccgatc	642	55°C	Jarraud et al. (2002)
	SEG-R	aaacttatatggaacaaaaggtagttc			
<i>seh</i>	SEH-F	caatcaactcatatgcgaaagcag	376	61.8°C	Jarraud et al. (2002)
	SEH-R	catctaccaaacattagcacc			
<i>sei</i>	SEI-F	ctcaagtgatattggttagg	577	61.8°C	Jarraud et al. (2002)
	SEI-R	aaaaaacttacagcagtcctctc			

pH 8.3; Bio-Rad, USA). A 100bp ladder (New England Biolabs, USA) was used for reference. The gel was stained in a solution of Gel Red 3X (Biotium, USA) for 30 minutes and visualized by a Gel Doc™ EZ System (Bio-Rad, USA). Positive controls used for comparison included *Staphylococcus aureus* FRI 996 (*sea*, *seb*, and *sed*), *S. aureus* ATCC 19095 (*sec*, *seh*, and *sei*), *S. aureus* ATCC 27664 (*see*), and *S. aureus* ATCC 23235 (*seg*).

Descriptive statistics were used (M S. Exel®).

## RESULTS

### *Staphylococcus* identification

One hundred fifty (150) isolates of *Staphylococcus* spp. were found in the 30 analyzed samples of buffalo milk. Using the coagulase test, 93 isolates (62%) were identified as producers of the coagulase enzyme *Staphylococcus* (CPS), and of these, 38 (40.9%) were isolated from the milk samples from Group 1 and 55 (59.1%) from Group 2. Of the total CPS, 68 (73.1%) were identified as *S. aureus*. In addition, 57 (38%) isolates were identified as coagulase-negative *Staphylococcus*.

### Antimicrobial susceptibility testing

The antibiogram results indicated that 13 (76.47%) of the 17 antibiotics tested were sensitive to all isolates with 100% efficiency, being: cefquinome, danofloxacin, tetracycline, ampicillin, enrofloxacin, ceftiofur, sulfatrim, amoxicillin + clavulanic acid, cefalonium, ciprofloxacin, oxacillin, amoxicillin, novobiocin + penicillin G procaine. The other tested antibiotics (cephalexin, gentamicin, neomicin and ampicillin + colistin) showed 22.22% resistance for the first three and 11.11% for the last one.

### Detection of enterotoxin-encoding genes

Thirty randomly selected *Staphylococcus aureus* isolates were submitted to encoding gene detection for classic staphylococcal toxins (*sea*, *seb*, *sec*, *sed* and *see*) and new enterotoxins (*seg*, *seh* and *sei*). Of these 30 isolates, enterotoxigenic genes were found in 12 isolates (40%), including 8 (66.7%) from animals in Group 1, and 4 (33.3%) from animals in Group 2. Genes involved in *sea* synthesis were amplified in 1 sample

**Table 2. Enterotoxin-encoding genes found in buffaloes belonging to Group 1 (early lactation animals) and Group 2 (late lactation animals)**

Enterotoxins <sup>a</sup>	n (%)	Group 1	Group 2
A	1 (8.3)	0	1
H	5 (41.7)	0	5
I	9 (75)	3	6
Total	12	6	6

<sup>a</sup> The presence of genes in association were observed in 3 samples among the enterotoxins found, specifically A+I in 1 sample, and H+I in 2 samples.

(3.33%, Group 2), the *seh* gene was amplified in 5 samples (16.6%, Group 1), and the *sei* gene was amplified in 9 samples (30.0%), being 2 from Group 1 and 7 from Group 2. Moreover, 3 samples (25%) were amplified for 2 genes in association (1 sample for *sea* plus *sei* and 2 samples for *seh* plus *sei*) as shown in Table 2 and Figure 1. Regarding the other *seb*, *sec*, *sed*, *see* and *seg* genes, 18 (60%) were not amplified for any isolates in this study.

## DISCUSSION

A lack of strict hygienization of equipment and animal health for food production of animal origin is considered as the main source of food contamination, since these deficiencies can lead to the cultivation of enterotoxin-producing bacteria. *Staphylococcus aureus* isolated from raw buffalo milk presented most of the *sei* and *seh* genes for staphylococcal enterotoxins; this is important because these genes are capable of causing food poisoning outbreaks and only a few of these genes have been studied for this species.

Antibiotic resistance found in this study was also the same as those found by all opportunists as etiologic agents of mastitis, and similar to the results found by Barros et al. (2013). The antibiotic widely used in the treatment of mastitis is tetracycline, which showed 100% efficiency in this study, where all isolated samples of *Staphylococcus* spp. were sensitive to the same active principle. Gentamicin and neomycin, which

has shown high efficacy in vitro against mastitis agents in different studies, can be ineffective, especially when its use is frequent and inappropriate (Brito et al. 2001), and which may have occurred in animals of the present study which showed resistance to these active principles. Antimicrobial therapy is one of the main tools for controlling mastitis in herds, enabling treatment to be performed with greater efficiency and safety, but it is important to emphasize the importance of assessing antimicrobial susceptibility in vitro for each case prior to indication of treatment, since each property has a different reality and differences in milking hygiene.

Studies of enterotoxin-encoding gene expression in *Staphylococcus aureus* strains obtained from raw milk samples of different animal species have demonstrated a large variability among isolates. Such variation included differences in the presence or absence of genes and the diversity of the identified types, as well as differences in the number of strains producing staphylococcal enterotoxins and the types of enterotoxins produced (Paulin et al. 2012). For the *S. aureus* strains isolated in the present study, the *sei* gene was the most commonly found, followed by the *seh* gene and *sea*. A low occurrence (3.33%) of classic enterotoxigenic *S. aureus* isolates was observed in this study, with the *sea* gene being the only classic staphylococcal enterotoxin gene detected. Silva et al. (2013) found that the genes involved in the synthesis of *seb*, *sec*, *sed* and *see* were not amplified in any of the isolates of mastitic milk from dairy cows in Brazil.

Oliveira et al. (2011) and Rahimi & Alian (2013) reported the presence of the *sea* and *sea* and *sed* classic staphylococcal enterotoxin genes, respectively, when characterizing *Staphylococcus* spp. isolated from buffalo milk, which is corroborated by the current results. A pioneering study of toxigenic *S. aureus* in milk from buffaloes with mastitis was described by Bonna et al. (2004), who showed that only enterotoxin A was detected among the classic staphylococcal enterotoxins, and which were observed in 51.6% of the studied herds.

Similar to the present study, Luz (2009) also did not find the presence of the *seb*, *sec*, *sed* and *see* genes; however, at least 1 of the enterotoxin-encoding H and I genes was detected in 93.6% of the *Staphylococcus aureus* strains, and the presence of *seh* plus *sei* (1.1%) genotype association was found among the isolates which were positive for the toxigenic *seg*, *seh*, *sei*, and *sej* genes. The absence of genes for classic toxins and the presence of genes for other toxins may be associated with the duration and geographical distribution of the toxigenic isolates. In fact, according to Luz (2009), the gene profiles for staphylococcal enterotoxins seem to be variable among different years and geographical origins.

The occurrence of staphylococcal enterotoxins found in 40% of the isolated *Staphylococcus aureus* in the present study is high compared to Ferreira et al. (2014), who reported an occurrence rate of 19.5%. Therefore, our results indicate that the presence of *S. aureus* which have enterotoxinogenic potential in raw buffalo milk may be a source of food poisoning.

## CONCLUSIONS

Several studies have investigated enterotoxins in cattle and goats, but there are few such studies on buffaloes. Additionally there is a lack of information on the occurrence of staphylococcal non-classical enterotoxin-encoding genes from milk. We highlight the need for further research and

greater attention to this species to estimate its impact on buffalo milk.

Although this study does not assess the expression of enterotoxin-encoding genes, its detection is directly indicated by the enterotoxigenic potential of these strains, highlighting the need for better quality control in animal production and health lines, as well as future studies to ensure the health of the population, as buffalo milk is exclusively intended for manufacturing dairy products and such transmissions of enterotoxins will also be present in the products.

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**Conflict of interest statement.**- The authors declare that they have no conflicts of interest.

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